

# Protective Effect of Melatonin on Kainic Acid Induced-Liver Damage and Immune Modulatory Cytokines

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## Abstract

Liver toxicity occurs when liver develops inflammation due to exposure to several toxic substances. The study was aimed to assess the liver damage induced by kainic acid and subsequently protective role of exogenous melatonin against the liver toxicity. Interestingly, kainic acid caused severe liver damage as evident from deleterious alterations in liver histology, increased lipid peroxide levels, decrease in the activities of liver anti-oxidant enzymes, DNA damage (adduct formation and sequence alterations), increased expression of cytokines like monocyte chemo-attractant protein-1, interleukin 6, interferon  $\gamma$  and decreased expression of interleukin 10. These changes were normalized by melatonin (0.5-1.0 mM, *in vitro*) or 10-20 mg/kg, (*in vivo*). The study included assessment of the expression of immune modulatory cytokine mediators using real time PCR in both *in vitro* and *in vivo* conditions in the mouse liver. DNA damage was also studied. Various oxidative stress parameters and liver histopathology was also evaluated. Melatonin's anti-kainic acid toxicity could be brought about by counter acting the influence of kainic acid on the levels of the cytokines, immune reactions and free radical production. This work suggests that melatonin receptors present could be mediate the hepatoprotective actions of melatonin therapy.

**Keywords:** Melatonin; Kainic Acid; Liver Damage; Immune Modulation; Cytokines

**Abbreviations:** KA: Kainic Acid; MLT: Melatonin; LPO: Lipid Peroxidation; GSH: Reduced Glutathione; GR: Glutathione Reductase; Gpx: Glutathione Peroxidase; SOD: Super Oxide Dismutase, IL10: Interleukin 10; IL6: Interleukin 6; MCP-1: Monocyte Chemo Attractant Protein-1; INF  $\gamma$ : Interferon Gamma; Mt DNA: Mitochondrial DNA

## Introduction

Liver toxicity or otherwise toxic hepatitis occurs when liver develops inflammation because of exposure to a toxic substance. Liver is a vital organ engaged in several vital functions, including removing most drugs and chemicals from the bloodstream, and breaking them down so that they can be quickly eliminated from the body. Breaking down toxins creates byproducts that can be highly damaging to the liver. Although the liver has a great capacity for regeneration, constant exposure to toxic substances can cause serious - and sometimes irreversible-harm. Excessive consumption of dangerous substances can overwhelm the liver's purification ability and the toxins may begin to take over, creating a poisonous environment in the liver.

Kainic Acid (KA) is a known neurotoxin which induces seizures. KA binds to glutamate receptor and causes excitotoxicity by influx of high levels of calcium ions into the cells [1]. This is associated with intense stimulation of microglia in the brain, monocyte infiltration, oxidative stress, induction of expression of genes such as IL-1 $\beta$ , TNF- $\alpha$ , iNOS and COX-2 and intense inflammatory responses [2-4].

Although KA is well known as a neurotoxin its effect on organs such as liver is not well studied. If intense oxidative stress occurs in liver it can cause liver damage. High levels of free radicals are known to damage hepatocytes and cause life threatening liver diseases [5]. KA influences immune system, generates excess free radicals and reduces the levels of anti-oxidant enzymes and induces oxidative stress not only in the brain but also in the liver and kidneys of mice [6]. Further, pro-inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$  have roles in hepato-toxicity [7]. The antioxidant and anti-inflammatory activity of

melatonin in the liver is already explored [8]. KA could severely damage liver tissue also. Studies were carried out to verify this hypothesis.

Melatonin, the hormone of darkness and messenger of the photoperiod, it is also well known to exhibit strong direct and indirect antioxidant properties. Melatonin has a powerful role in organ protection in numerous models of injury; these beneficial effects have been attributed to the hormone's intense radical scavenging capacity. Melatonin exhibits Hepato protective potential in various models of oxidative stress *in vivo*, and is a suitable experimental substance to reduce liver damage after sepsis, hemorrhagic shock, ischemia/reperfusion, and in numerous models of toxic liver injury [9-11].

Melatonin's influence on hepatic antioxidant enzymes and other potentially relevant pathways, such as nitric oxide signaling, hepatic cytokine and heat shock protein expression, are evaluated. Various studies demonstrate the functional relevance of melatonin acts as substance exhibiting for hepatoprotection [12].

KA causes an increase in the levels of 8-hydroxy-deoxyguanosine in brain and liver; melatonin reduces this increase [13]. Further, melatonin inhibits chronic ethanol administration-induced increase in the levels of lipid peroxides in several organs of rats including liver [14]. Melatonin attenuates alcoholic liver injury. It decreases serum and liver tissue inflammatory cytokines levels, tissue lipid peroxidation and neutrophil infiltration in liver [15]. It prevents many of the diabetic

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complications in liver, kidney and brain by reducing oxidative stress [16]. Previous investigators thought of interest to study the likely protective role of melatonin on KA induced liver damage, if any.

With respect to interleukin signaling, melatonin was reported to suppress the formation of pro-inflammatory cytokines such as tumor necrosis factor  $\alpha$ , interleukin (IL)-1, interleukin (IL)-1 $\beta$ , interleukin (IL)-6, as well as the cellular interleukin response protein, nuclear factor  $\kappa$ -light-chain-enhancer of activated B cells [17-22].

Interleukin (IL)-10 is known to reduce oxidative stress in liver. It can inhibit a range of macrophage function and it has been suggested that IL-10 synthesized during the course of liver inflammation and fibrosis may modulate Kupffer cells and subsequently progression of fibrosis [23].

IL-6 can induce hepatic inflammation and is involved in the pathogenesis of fibrosis and diseases of the liver [24]. It is the necessary cytokine for the induction of hepcidin during inflammation and that IL-6-hepcidin axis is responsible for the hypoferrremia of inflammation [25]. Exposure to INF $\gamma$  suppressed chemical hepatocarcinogenesis, despite overt liver injury [26]. Monocyte Chemoattractant protein-1 (MCP-1) levels were increased in liver from patients with fulminant hepatic failure. It supports a role for MCP-1 in the pathogenesis of human liver disease [27]. MCP-1 deficiency improved insulin resistance and hepatic steatosis in A-ZIP-Tg mice [28]. Deficiency of MCP-1 protects mice against alcoholic liver injury also [29]. MCP-1 secreted by FSC may participate in the recruitment and activation of monocytes at sites of liver injury [30].

The effect of KA on expression of IL-10, IL-6, MCP-1 and INF  $\gamma$  in liver is not known. Therefore, influence of KA on the expression of these cytokines in liver and the role of melatonin on the same was also studied.

The hypothesis is KA markedly stimulates certain immune reactions particularly, activation of macrophages/Kupffer cells through modulation of the expression of certain immune modulatory genes resulting in marked oxidative stress and subsequent DNA damage, apoptosis etc., of liver tissue. Melatonin counteracts the influence of KA on immune system and attenuates oxidative stress and liver damage.

## Methods

### Chemicals and reagents

Thiobarbituric Acid (TBA), Reduced Glutathione (GSH), Oxidized Glutathione (GSSG), Dithio-bis-2-nitrobenzoic Acid (DTNB), Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>), Disodium Hydrogen Phosphate (Na<sub>2</sub>HPO<sub>4</sub>), Sodium Dihydrogen Phosphate (NaH<sub>2</sub>PO<sub>4</sub>), Ethylene Triamine Tetra acetic Acid (EDTA), Agarose, Kainic acid and Melatonin were from Sigma Chemical Co., St Louis, Missouri, USA; RNase, ethanol, Bromophenol blue, Ethidium bromide and Taq Polymerase from Fermentas, USA; High sensitive 8-OHdG check ELISA kit from Control of Aging, Fukuroi, Japan. Mouse oligonucleotide primers for Interleukin-6 (IL 6), Interleukin-10 (IL 10), Interferon-gamma (INF  $\gamma$ ), Monocytes Chemoattractant Protein-1 (MCP-1) and glyceraldehyde phosphate dehydrogenase (GAPDH) were procured from Eurogentec, Belgium; RPMI-1640 from Himedia, Mumbai, India; RT2 SYBR green ROX q PCR master mix, RNeasy lipid tissue mini kit, Qiazol lysis reagent and RT2 first strands kit from Qiagen, Hilden, Germany. Mouse CD4+ selection kit from Stem Cell Technologies Inc, Canada; Mitochondrial DNA (mt DNA) extractor CT kit from WAKO Pure

Chemical Industries Ltd, Japan. 3H thymidine from BRIT, India. All the other chemicals used were of analytical grade and were purchased from qualified local vendors.

### Animals

Swiss albino mice of either sex aged 4 to 5 weeks, weighing 17-23 g were selected for this study. The animals were procured from the Division of Laboratory Animal Sciences of Biomedical Technology wing (BMT), Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram and maintained in a 12 h light and dark cycle at controlled environmental conditions of temperature (22  $\pm$  3°C) and humidity (50-70%). They were fed with standard pellet diet and provided water ad libitum.

All animals were handled humanely, without causing pain or distress and with due care for their welfare. The care and management of the animals comply with the regulations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India. All the animal experiments were carried out with prior approval from Institutional Animal Ethics Committee and in accordance with approved institutional protocol.

All experiments were carried out in the morning hours. During the experiments the mice were placed in individual cages. Animals received KA (to induce seizures) or different concentrations of MLT by intra peritoneal (i.p.) route. Animals were euthanized by cervical dislocation.

### Experimental design for *in vitro* studies

Six healthy Swiss albino mice were sacrificed by cervical dislocation. Liver and spleen were dissected out immediately and washed with sterile Phosphate Buffered Saline (PBS). The liver thus obtained was homogenized with Polytron homogenizer at 900 rpm in physiological saline to obtain 25% homogenate [31]. The splenocytes were collected by disrupting the isolated spleens in PBS.

The treatment of different homogenate with KA and MLT were carried out as per the method of [31,32]. Briefly, the tissue homogenate was divided in to several groups; each group contained 1 ml of the liver homogenate in triplicate. The first group was kept as normal control, treated with 1mM of physiological saline; homogenates in group II were treated with 1 mM of KA; homogenates in group III were treated with 1 mM of MLT; homogenates in groups IV, V and IV were treated with 1 mM of KA+0.25 mM of MLT, 1 mM of KA+0.5 mM of MLT and 1 mM of KA+1 mM of MLT respectively. All groups of homogenates were incubated at 37°C for 15 min and used to determine various biochemical and molecular parameters.

### Experimental design for *in vivo* studies

Thirty two healthy Swiss albino mice (17-23 g) were selected and divided in to eight group of four each. The KA, MLT and KA+MLT combination doses were selected as described elsewhere [31]. The group I served as normal control received physiological saline, animals in group II received 45 mg/kg of KA in 0.5 ml of physiological saline, animals in group III received 20 mg/kg of MLT in 0.5 ml of physiological saline, animals in group IV, V, VI received simultaneous administration of 45 mg/kg of KA+5 mg/kg MLT, 45 mg/kg of KA+15 mg/kg MLT, 45 mg/kg of KA+20 mg/kg MLT respectively. Animals in group VII and VIII received 45 mg/kg of KA+5 mg/kg MLT and 45 mg/kg of KA+20 mg/kg MLT respectively, where MLT was administered 15 min prior to KA administration (pre-treatment). All animals received the vehicle/KA/MLT intra peritoneal route (in 0.5 ml physiological saline/ animal),

and were observed for 1 h to determine the severity of seizure [31,32]. After 1 h all animals were sacrificed by cervical dislocation and liver was carefully dissected out and homogenized to determine various biochemical and molecular parameters. Spleen was removed for the preparation of splenocytes.

### Measurement of Lipid Peroxidation (LPO) and other antioxidant parameters of liver (*in vitro* and *in vivo* experiments)

Different groups of liver homogenates obtained from the *in vitro* and *in vivo* experiments were used to determine the levels of tissue lipid peroxidation and different antioxidant parameters. Briefly, 500  $\mu$ l of respective liver homogenates in 1 mM of PBS (PH-7.4) were centrifuged at 3500 rpm for 10 minutes at 4°C and supernatants were collected for the estimation of total protein, LPO and different antioxidant parameters using standard protocols. Estimation of protein was done by the method of [29] with bovine serum albumin as standard. Estimation of LPO was done as per the method of [30], the amount of malondialdehyde (MDA) formed was measured spectrophotometrically at 532 nm. GSH levels were determined by the method [33] and the amount of GSH was expressed as n mole / mg protein. Determination of glutathione reductase activity was performed as described [34]. Activity of GPX was assayed by the method described [35]. Activity of SOD was assayed as described elsewhere [36].

### Real time PCR analysis for determining m-RNA of specific cytokines

Liver tissue (100 mg) from the *in vitro* and *in vivo* experimental groups was used to extract total m-RNA following the manufacturer's protocol, using Trizol reagent (Sigma, USA). 150 ng of m-RNA were used for c-DNA synthesis of IL-10, IL- 6, MCP-1, INF $\gamma$  and GAPDH in a reaction volume of 20  $\mu$ L using RT2 first strand Kit (Qiagen, Germany) and the syntheses were carried out on the Eppendorf master cycler, Germany. The mouse oligo nucleotide forward and reverse primer sequence used to determine specific m-RNA gene expressions were: f- GCGTGGGGACAGCCGCATCTT and r-ATCGGCAGAAGGGGCGGAGA for GAPDH (accession no. BC 023196.1). The real time PCR reaction was carried out with RT2 SYBR green ROX q PCR master mix of total reaction volume of 25  $\mu$ l, real time PCR amplifications were done using a Chromo 4 System, Bio-Rad (MJ Research, CA) for 40 cycles as per manufacturer's protocol. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the house keeping gene. The level of gene expression is reported as the ratio between the mRNA level of the target gene and the GAPDH, a reference gene using the comparative 2 $\Delta\Delta$ Ct method [37].

### Mitochondrial DNA (mt DNA) isolation and amplification of cytochrome b DNA

Mitochondrial DNA (mt DNA) was isolated from liver tissues from *in vitro* and *in vivo* experimental groups individually as per the manufacturer's protocol (WAKO Pure Chemical Industries Ltd, Japan). The quantity and quality of the isolated mt DNA were estimated spectrophotometrically. Cytochrome b DNA of the isolated mtDNA from *in vitro* and *in vivo* experimental groups were amplified using mouse Cytochrome b specific forward primer (f-ACACAAAGGTTTGGTCCTGGCCTTA) and reverse primer (r-CGTATGACCGCGTGGCTGG) (Eurogenetic, Belgium, Accession no. HQ675031.1) at concentration of 100 ng/ $\mu$ l per reaction mixture. PCR of mtDNA was carried out as per standard conditions

[38] in Eppendorf master cycler; Germany. Purity and integrity of the amplified products were checked by purity factor (260/280 nm) and agarose gel electrophoresis.

S.No	Primer	Primer Sequence	Accession No.
1	Interleukin 10 (IL-10)	f- CCAAGTCGGCCAGACCCACAT r-GGCCATGCTTCTCTGCCTGGG	NM 010548.2
2	MCP-1)	F- AGCACCAGCACCAGCCAACCTC R- TGGATGCTCCAGCCGGCAACT	NM 011333.3
3	Interleukin 6 (IL-6)	F-GAGAGCATTGGAAATTGGGGTA R- GAGAGCATTGGAAATTGGGGTA	NM 031168.1
4	ING- $\gamma$	F- AGGCTCACGTCACCAAGTCCC R-GTCTCGAAAGCTACGTGGGAGG	M 34815.1
5	GAPDH	F-GCGTGGGGACAGCCGCATCTT R-ATCGGCAGAAGGGGCGGAGA	BC 023196.1

### Analysis of liver tissue mitochondrial DNA (mtDNA) adducts formation

8 Hydroxy, 2' deoxyguanosine (8-OHdG) in liver mt DNA digests *in vitro* and *in vivo* experimental groups were determined by a competitive immunosorbent assay using high sensitive 8-OHdG kit following manufacturer's protocol (Japan Institute for the Control of Aging, Fukuroi, Japan). The absorbance of the reaction mixture was read at 450 nm using ELISA microplate reader (Asys Expert plus, Austria). The unknown concentration of 8-OHdG in each sample was determined by comparing it with a standard curves of 8-OHdG.

### Sequence analysis of cytochrome b mitochondrial DNA (mtDNA)

The amplified cytochrome b DNA obtained from the PCR of mouse mtDNA from *in vitro* and *in vivo* experimental groups were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA sequencing was done by Sanger's dideoxy chain termination method [39]. Cytochrome b mtDNA products were sequenced using mouse cytochrome b forward and reverse primers in AB1 prism 3730 Genetic analyzer DNA sequencer with a Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems Japan Co., Ltd., Tokyo, Japan). ABI Sequence Scanner was used for sequencing and alignment of sequence was done using Bioedit software. After sequence analysis and alignment, using sequence navigator program ver. 2.1, all sequences were submitted to NCBI website: (<http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/>) and BLAST sequence similarity search was conducted.

### Tritiated (3H) thymidine incorporation assay using isolated splenocytes and CD4+ lymphocytes

Spleens excised from *in vitro* and *in vivo* experimental animals were used for the isolation of splenocytes and CD4+ lymphocytes to study the cell proliferation by tritiated thymidine incorporation assay in cell culture. Viability of splenocytes and isolated CD4+ cells were assessed using trypan blue dye exclusion method [40]. Single cell splenocytes suspension was used to isolate CD4+ cells according to the protocol supplied with the kit in an automated cell separator (ROBOSEP). Total splenocytes and CD4+ lymphocytes were cultured at a density of 2 $\times$ 10<sup>6</sup> and 8 $\times$ 10<sup>5</sup> cells/ml respectively in a 96 well plate with RPMI-1640 medium supplemented with 10% FBS, streptomycin (100  $\mu$ g/ml) and penicillin (100 units/ml) for 24 h at 37°C in a CO<sub>2</sub> incubator. After 24 h of incubation KA alone, MLT alone and various concentrations of KA and MLT combinations (as mentioned in experimental design *in vitro* and *in vivo*) were added in the respective well of the culture plates. Cells without any treatment served as control. After 48 h of



incubation 3H-thymidine at a concentration of 1  $\mu$ Ci/mL was added to the cultured cells in each well and incubated further for 24 h at 37°C. Cells were harvested after 72 h and radioactivity in terms of counts per minute (cpm) were measured by liquid scintillation counter.

### Histo-pathological evaluation

The fixed liver tissues were processed in a tissue processor (Leica ASP 300) and sectioned (5  $\mu$ m thin sections) using a rotary microtome (RM2255) and stained with hematoxylin and eosin (H&E). Stained sections were examined under a light microscope (Axio ImagerZ1, Carl Zeiss). Extent of tissue response and inflammation surrounding the tissue were semi quantitatively measured. Extent of fibrous capsule formation was measured quantitatively. Different types of inflammatory cells namely polymorphonuclear cells, lymphocytes, eosinophils, plasma cells, macrophages and foreign body type giant cells were measured quantitatively. Neovascularisation, degeneration and necrosis were determined qualitatively. Associated parameters like fatty infiltration, granuloma formation and tissue growth were also assessed.

### Statistical analysis

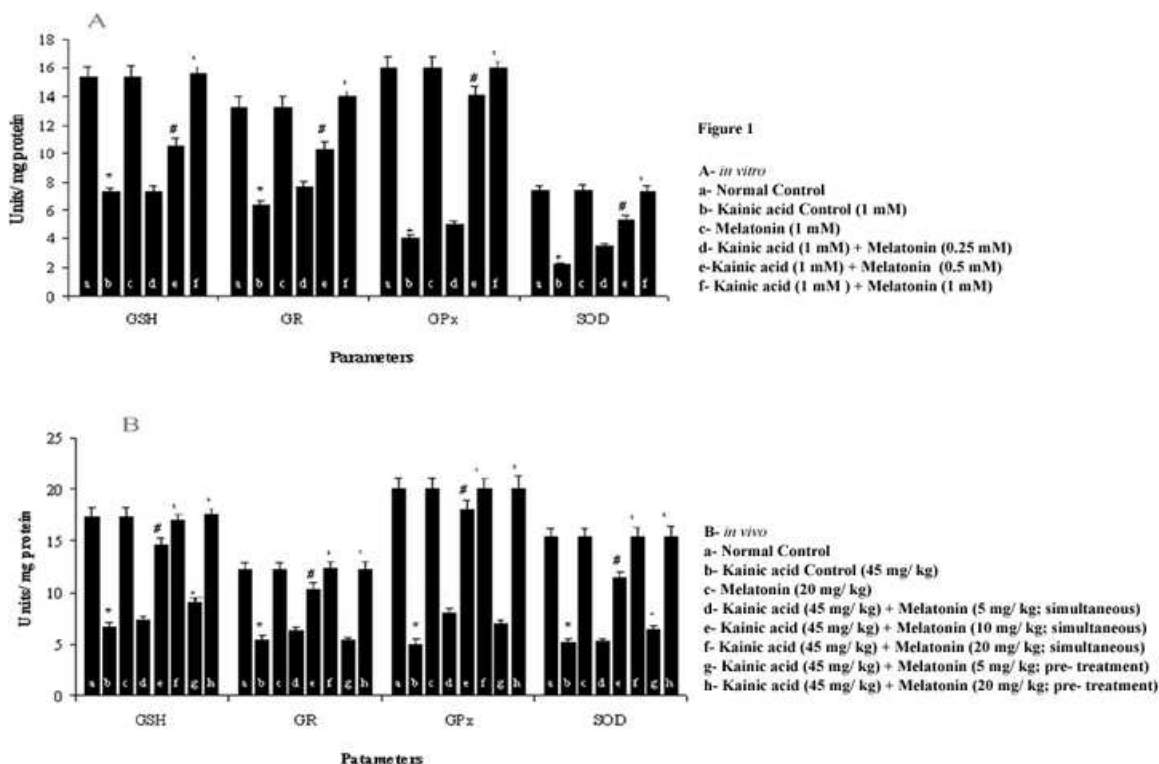
All the samples were run in triplicates; Statistical comparison was done with three or more groups using one-way analysis of variance

(ANOVA) followed by Dunnett's test. P values <0.001 and <0.05 were considered significant. Kainic acid control group was compared with normal control group. Kainic acid- melatonin treated combination groups were compared with the Kainic acid control group to assess the statistical significance using ANOVA.

## Results

### Protection of MLT on KA induced oxidative stress

*In vitro* KA treatment resulted in a drastic reduction in the levels of liver reduced glutathione and in the anti-oxidant enzymes activities (glutathione reductase, glutathione per oxidase and superoxide dismutase) which is evident from Figure 1A. MLT (1 mM) alone treatment did not influence these parameters. MLT co-treatment with KA attenuated KA-induced reduction in these anti-oxidant parameters in a concentration dependent manner. MLT considerably reversed KA-induced reduction in the anti-oxidant parameters at higher concentrations (0.5 and 1 mM). MLT completely prevented KA's effect on these parameters compared to KA control (1 mM). Interestingly Figure 1B, *in vivo* treatment also showed almost similar effects of KA on these parameters. MLT (simultaneous) treatment (10 or 20 mg/kg) completely reversed KA (45 mg/kg)-induced marked reduction in the levels of the anti-oxidant parameters when compared to KA



**Figure 1:** A- *In vitro* effect of melatonin on kainic acid- induced reduction in mouse liver glutathione and anti-oxidant enzymes. Normal control, b- Kainic acid control (1 mM), c- Melatonin (1 mM), d- Kainic acid (1 mM)+Melatonin (0.25 mM), e-Kainic acid (1 mM)+Melatonin (0.5 mM), f- Kainic acid (1 mM)+Melatonin (1 mM). GSH-reduced glutathione in nmol/mg protein; GR- glutathione reductase (units of enzyme activity/mg of protein). GPx- glutathione peroxidase (units of enzyme activity/mg of protein); SOD- superoxide dismutase (units of enzyme activity/mg of protein). Values are mean  $\pm$  S.D.; \*P<0.005 (compared to respective normal control); #P<0.005, #P<0.05 (compared to Kainic acid control). n=4 in each group. B- *In vivo* effect of melatonin on kainic acid- induced reduction in mouse liver glutathione and anti-oxidant enzymes. a- Normal control, b- Kainic acid control (45 mg/kg), c- Melatonin (20 mg/kg), d- Kainic acid (45 mg/kg)+Melatonin (5 mg/kg; simultaneous), e- Kainic acid (45 mg/kg)+Melatonin (10 mg/kg; simultaneous), f- Kainic acid (45 mg/kg)+Melatonin (20 mg/kg; simultaneous), g- Kainic acid (45 mg/kg)+Melatonin (5 mg/kg; pre-treatment), h- Kainic acid (45 mg/kg)+Melatonin (20 mg/kg; pre-treatment). GSH-reduced glutathione in nmol/mg protein; GR- glutathione reductase (units of enzyme activity/mg of protein); GPx- glutathione peroxidase (units of enzyme activity/mg of protein); SOD- superoxide dismutase (units of enzyme activity/mg of protein). Values are mean  $\pm$  S.D.; \*P < 0.005 (compared to respective normal control); #P<0.005, #P<0.05 (compared to Kainic acid control). n=4 in each group.

control (45 mg/kg). However, a low dose (5 mg/kg) of MLT did not reverse significantly, on the effects induced by KA on these parameters. Simultaneous treatment of MLT (5 or 20 mg/kg) and KA was found to be not significantly different from MLT administration 15 min prior to KA [pre- treatment] (Figure 1B).

As shown in Table 1, the levels of lipid peroxides in the liver were increased by KA treatment both *in vivo* and *in vitro* conditions. MLT treatment attenuated the same (*in vitro* and *in vivo*) in a concentration dependent manner. At a lower concentration (0.25 mM, *in vitro* or 5 mg/kg, *in vivo*) MLT partially counteracted the effect of KA on the levels of lipid peroxides which was not significant as compared to higher concentrations of MLT co treated when compared with the

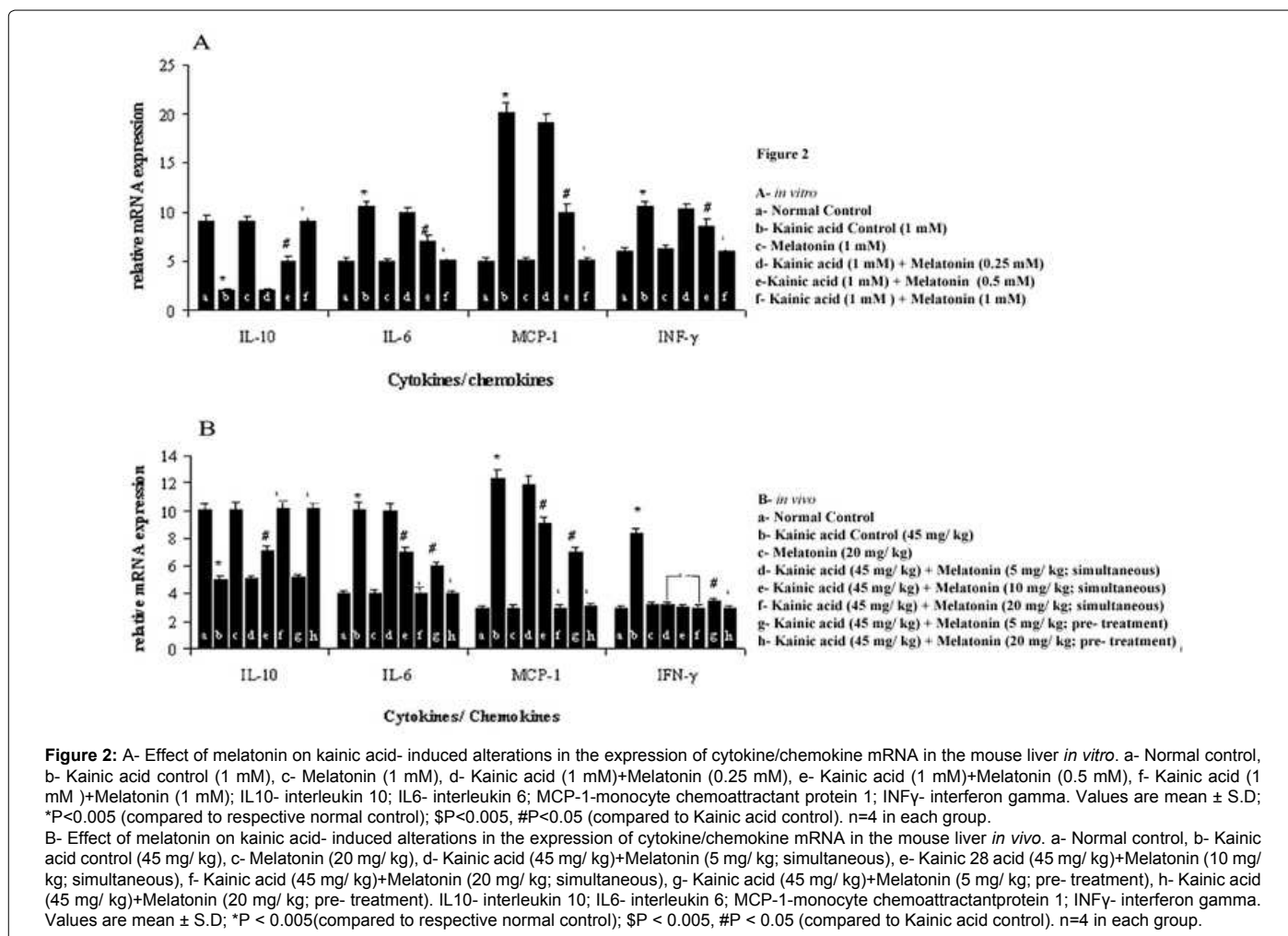
KA alone controls both *in vitro* and *in vivo*. In higher MLT co treated concentrations KA induced effects was fully normalized the levels of lipid peroxides in the KA treated (*in vitro* and *in vivo*) liver (Table 1) when compared with the KA alone controls both *in vitro* and *in vivo*.

### Effect of MLT on KA-Induced alterations in the mRNA expressions of IL-6, IL-10, MCP-1 and INF-γ using real time PCR

From Figure 2A, it is evident that *in vitro* treatment of liver tissue with KA resulted in a remarkable decrease in the levels of IL-10 mRNA expression, whereas 4 fold increases in MCP-1 mRNA expression was observed when compared to normal control. IL-6 and INF γ mRNA expressions were also increased. MLT attenuated these changes in

<i>In vivo</i> Treatments	nmol of MDA/mg Protein	<i>In vitro</i> Treatments	nmol of MDA/mg Protein
control	10.0 ± 0.5	control	14.2 ± 0.3
KA (45 mg/ kg)	23.0 ± 0.3*	KA (1 mM)	22.3 ± 0.5*
MLT (20 mg /kg)	10.2 ± 0.7 \$	MLT (1mM)	14.3 ± 0.4\$
KA (45 mg/ kg)+MLT (5 mg/kg, silmultaneous)	22.8 ± 0.3	KA (1 mM)+MLT (0.25 mM)	22.4 ± 0.6
KA (45mg/ kg)+MLT (10 mg/kg, silmultaneous)	13.3 ± 0.5 #	KA (1 mM)+MLT (0.5 mM)	16.5 ± 0.2 #
KA (45mg/ kg)+MLT (20 mg/kg, silmultaneous)	10.0 ± 0.4 \$	KA (1 mM)+MLT (1 mM)	14.0 ± 0.2 \$
KA (45mg/ kg)+MLT (5 mg/kg, pre- treatment)	14.8 ± 0.5 #	--	--
KA (45mg/ kg)+MLT (20 mg/kg, pre- treatment)	10.0 ± 0.1 \$	--	--

**Table 1:** Effect of melatonin on lipid peroxidation in liver tissue treated with kainic acid (*in vitro* and *in vivo*). KA- kainic acid, MLT- melatonin; Values are mean ± S.D; n= 4; \*P<0.005(compared to the Normal Control group); \$P<0.005, #P<0.05 (compared to Kainic acid control).



**Figure 2:** A- Effect of melatonin on kainic acid- induced alterations in the expression of cytokine/chemokine mRNA in the mouse liver *in vitro*. a- Normal control, b- Kainic acid control (1 mM), c- Melatonin (1 mM), d- Kainic acid (1 mM)+Melatonin (0.25 mM), e- Kainic acid (1 mM)+Melatonin (0.5 mM), f- Kainic acid (1 mM)+Melatonin (1 mM); IL10- interleukin 10; IL6- interleukin 6; MCP-1-monocyte chemoattractant protein 1; INFγ- interferon gamma. Values are mean ± S.D; \*P<0.005 (compared to respective normal control); \$P<0.005, #P<0.05 (compared to Kainic acid control). n=4 in each group.

B- Effect of melatonin on kainic acid- induced alterations in the expression of cytokine/chemokine mRNA in the mouse liver *in vivo*. a- Normal control, b- Kainic acid control (45 mg/ kg), c- Melatonin (20 mg/ kg), d- Kainic acid (45 mg/ kg)+Melatonin (5 mg/ kg; simultaneous), e- Kainic acid (45 mg/ kg)+Melatonin (10 mg/ kg; simultaneous), f- Kainic acid (45 mg/ kg)+Melatonin (20 mg/ kg; simultaneous), g- Kainic acid (45 mg/ kg)+Melatonin (5 mg/ kg; pre- treatment), h- Kainic acid (45 mg/ kg)+Melatonin (20 mg/ kg; pre- treatment). IL10- interleukin 10; IL6- interleukin 6; MCP-1-monocyte chemoattractantprotein 1; INFγ- interferon gamma. Values are mean ± S.D; \*P < 0.005(compared to respective normal control); \$P < 0.005, #P < 0.05 (compared to Kainic acid control). n=4 in each group.

mRNA expression concentration dependent manner. MLT (0.5 or 1 mM) combination with KA completely prevented KA-induced changes in the expression of the mRNAs of the cytokines/chemokines compared to KA control. However, at a low concentration (0.25 mM) MLT only partially prevented KA-induced changes in the m-RNA levels compared to KA control.

As shown in Figure 2B, *in vivo* treatment of KA showed almost similar pattern of changes as compared with *in vitro* treatment on the expression of the mRNAs of the cytokines. KA reduced the mRNA levels of IL-10 to almost 20% of normal control. MLT (5-20 mg/kg) prevented this drastic reduction in a concentration dependent manner; at 20 mg/kg level, it completely prevented the decrease in IL 10 expression induced by KA. mRNA expression levels for MCP-1, IL-6 and INF $\gamma$  were markedly increased by KA treatment and MLT (10 or 20 mg/kg) combination with KA fully counteracted this increase in the mRNA levels. Pronounced 4 fold increase in MCP 1 expression was noticed, these changes were fully abolished by MLT (20 mg) However, and at 5 mg/kg dose MLT's influence on the KA-induced increase in the levels MCP 1 of mRNAs was partial when compared to KA control. Simultaneous treatment of MLT with KA did not show any significantly effect (Figure 2B).

### Protection of MLT on KA-induced liver mtDNA adduct formation

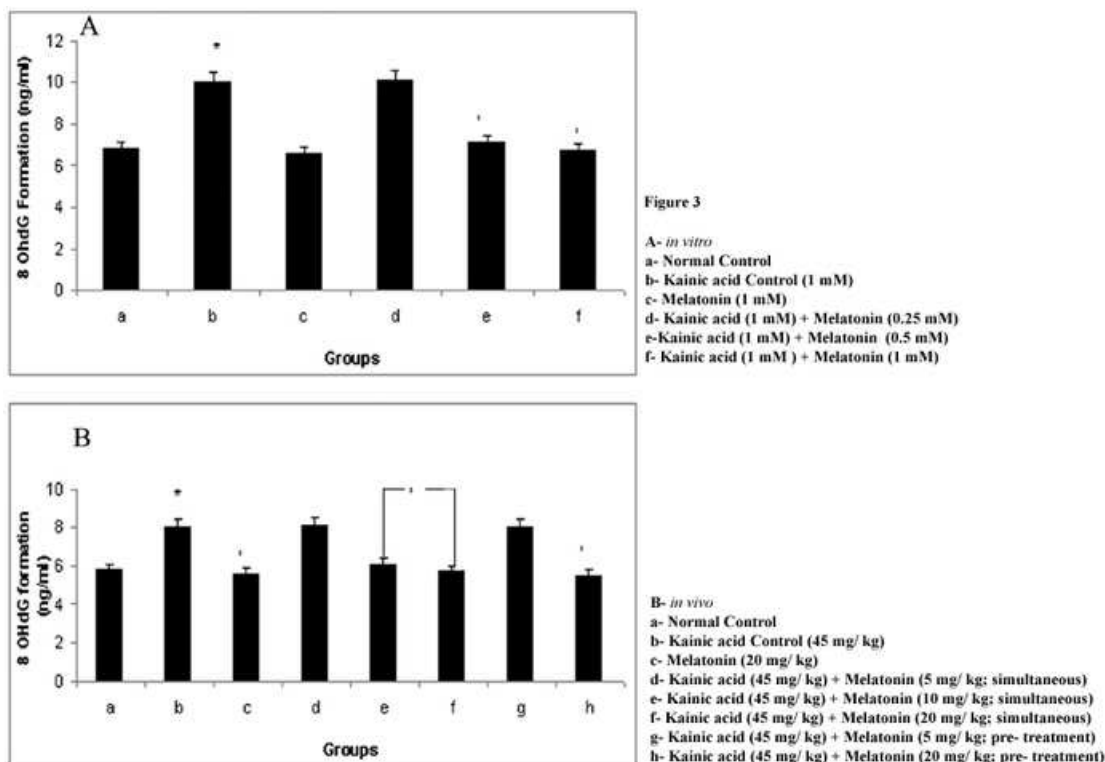
*In vitro* and *in vivo* studies showed that, KA treatment increased liver mtDNA adduct formation (Figures 3A and B). MLT protected

the DNA from increase in the mtDNA adduct formation caused by KA in a concentration dependent manner. These changes were more pronounced under *in vivo* conditions. Simultaneous treatment of MLT with KA and 15 min pre-treatment of MLT (*in vivo*) did not differ significantly in its effect on the attenuation of mtDNA adduct formation (Figure 3B) compared to KA control.

### Attenuation of KA-induced liver mitochondrial cytochrome b gene sequence alterations by MLT

The sequence alignment of the mitochondrial cytochrome b gene isolated from liver tissues of *in vivo* experimental animals showed significant increase in base mismatching and lesser DNA sequence alignment in KA alone treated group. Maximum DNA sequence alignment was observed in MLT alone treated group, while all other groups did not show any significant percentage deviation of sequence alignment from the control group (Figure 4). The MLT treated (10 or 20 mg/kg) prevented such mismatches. Simultaneous treatment of MLT with KA and 15 min pre-treatment of MLT (*in vivo*) did not differ significantly in its effect on the attenuation of mtDNA adduct formation (Figure 4). Line 'C' clearly depicts that KA administration *in vivo* in mice resulted in the Sequence alterations as well as some base pair deletions in the cytochrome b genome. Also, it is evident from the Figure 4a (lines D-H) MLT in combination with KA prevented these alterations *in vivo*.

However lower concentrations of KA and MLT showed moderate changes in the DNA sequence when compared with KA (1mM) alone



**Figure 3:** A- *In vitro* effect of melatonin on mtDNA adduct formation in kainic acid treated liver issue. Normal control, b- Kainic acid control (1 mM), c- Melatonin (1 mM), d- Kainic acid (1mM)+Melatonin (0.25 mM), e- Kainic acid (1 mM)+Melatonin (0.5 mM), f- Kainic acid (1 mM)+Melatonin (1 mM); Values are mean  $\pm$  S.D \*P<0.005 (compared to respective normal control); \$P<0.005, #P<0.05 (compared to Kainic acid control).n=4 in each group.n=3 in each group. B- *In vivo* effect of melatonin on mtDNA adduct formation in kainic acid challenged liver tissue.a- Normal control, b- Kainic acid control (45 mg/kg), c- Melatonin (20 mg/kg), d- Kainic acid (45 mg/kg)+Melatonin (5 mg/kg; simultaneous), e- Kainic acid (45 mg/kg)+Melatonin (10 mg/kg; simultaneous), f- Kainic acid (45 mg/kg)+Melatonin (20 mg/kg; simultaneous), g- Kainic acid (45 mg/kg)+Melatonin (5 mg/kg; pretreatment), h- Kainic acid (45 mg/kg)+Melatonin (20 mg/kg; pre-treatment).Values are mean  $\pm$  S.D.\*P<0.005(compared to respective normal control); \$P<0.005, #P<0.05(compared to Kainic acid control). n=4 in each group.

treated group. Interestingly, higher concentrations of KA and MLT combination groups, both *in vitro* and *in vivo* showed significant protection to DNA bases in cytochrome b genome when compared with KA (1mM) alone treated group (Figure 4).

**Effect of MLT on 3H-Thymidine incorporation into KA-challenged splenocytes and CD4+ lymphocytes (*in vitro* and *in vivo* treatments)**

*In vitro* KA (1 mM) treatment reduced 3H-Thymidine incorporation in both total splenocytes and CD4+ lymphocytes. MLT in a concentration dependent manner attenuated this reduction in 3H-Thymidine incorporation. KA-induced inhibition of 3H-Thymidine incorporation was suppressed by 0.25 mM MLT while higher concentrations (0.5 and 1 mM) in combination with KA of MLT fully abolished the effect of KA on 3H-Thymidine incorporation (Figure 5A) compared to KA control.

A pattern of change *in vivo* was very similar to that of *in vitro* treatment. KA substantially decreased 3H-Thymidine incorporation both in total splenocytes and CD4+ lymphocytes. MLT co-administration at a dose of 10 or 20 mg/kg completely abolished the inhibitory effect of KA on the 3H-Thymidine incorporation in both splenocytes and CD4+ lymphocytes. However, 5 mg/kg MLT did not influence KA-induced reduction in 3H-Thymidine incorporation into CD4+ lymphocytes while it marginally reduced the effect of MLT on the same in the case of splenocytes (Figure 5B) compared to KA control. Simultaneous as well as pre-treatment with MLT showed the same level

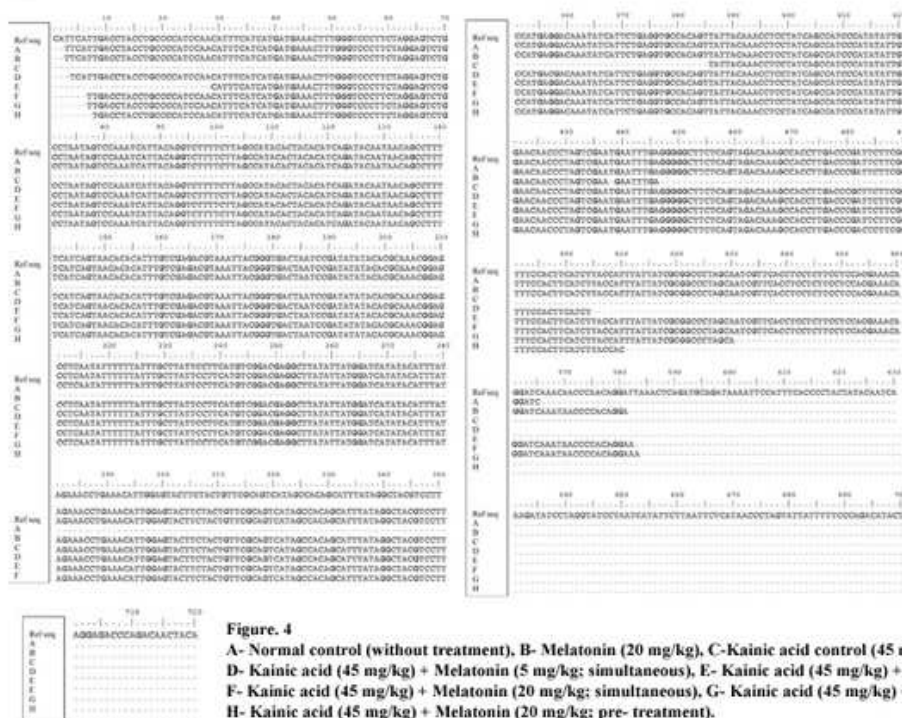
of effect on KA-induced reduction in 3H-Thymidine incorporation in to the cells compared to KA control.

**Histopathology evaluation of liver**

Histopathological evaluation with hematoxylin and eosin staining revealed that Control (no treatment) liver sections showed normal architecture and mild fatty change only (Figure 6A). Whereas KA alone (45 mg/kg) treated liver tissues sections of liver showed marked perivascular lymphocytic infiltrate, granular cell change and ballooning degeneration of hepatocytes (Figure 6B). MLT alone (20 mg/kg) treated liver tissues sections of liver showed normal architecture and mild fatty change similar to that of normal control liver sections (Figure 6C). The doses KA (45 mg/kg)+MLT (5 mg/kg) and KA (45 mg/kg)+MLT (10 mg/kg) showed some perivascular lymphocytic infiltrate, granular cell change and ballooning degeneration of hepatocytes with mild fatty change respectively (Figures 6D and E). However higher dose of co treated MLT i.e, Kainic acid (45 mg/kg)+Melatonin (20 mg/kg) such architectural liver changes were absent (Figure 6F) compared to KA control (Figure 6B).

**Discussion**

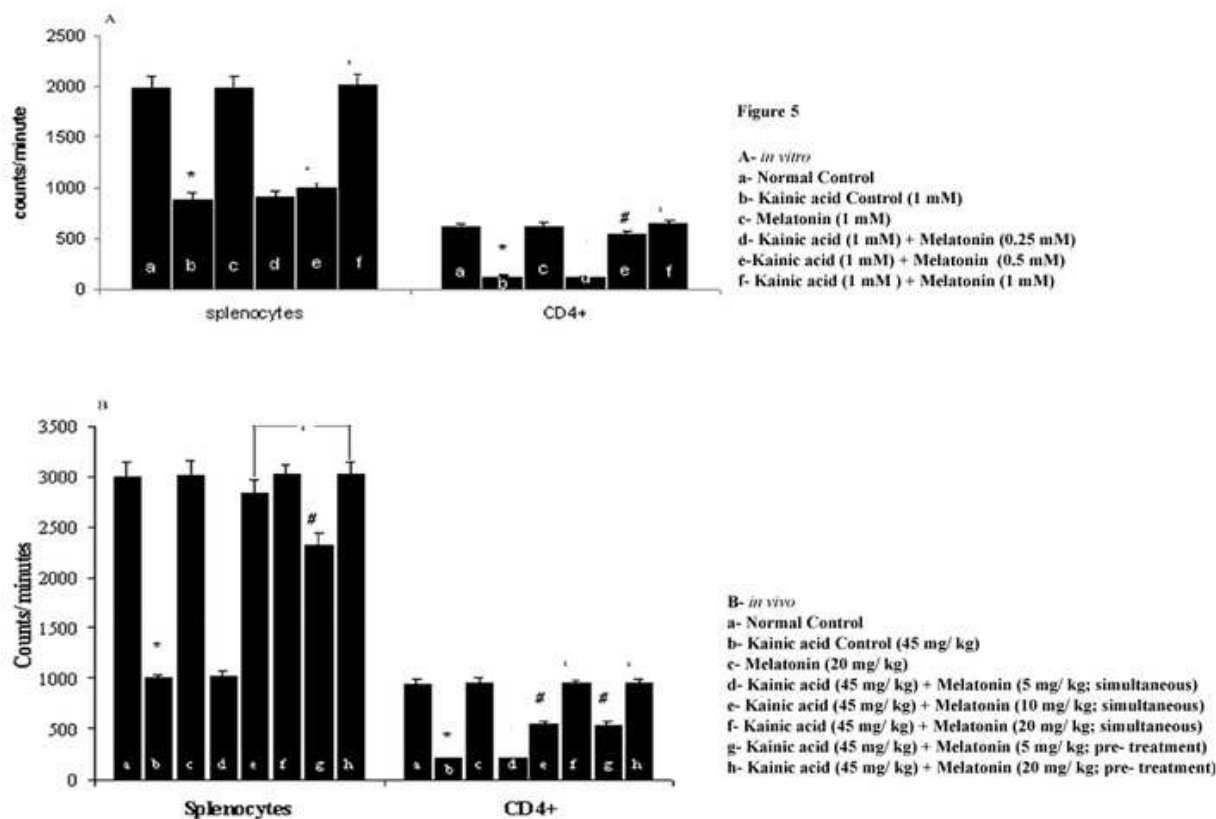
The study revealed that KA could induce severe liver damage, as evidenced from deleterious alterations in liver histo-architecture, lipid peroxides and anti-oxidant enzyme levels, and melatonin protected mice against KA-induced hepatotoxicity. Previous histopathological studies on brain tissue have shown that KA induces oxidative stress in brain, in form of neuronal lesions [41]. The present study showed that KA induced liver damage causing hepatocyte degeneration. KA also



**Figure 4**  
 A- Normal control (without treatment), B- Melatonin (20 mg/kg), C- Kainic acid control (45 mg/kg), D- Kainic acid (45 mg/kg) + Melatonin (5 mg/kg; simultaneous), E- Kainic acid (45 mg/kg) + Melatonin (10 mg/kg; simultaneous), F- Kainic acid (45 mg/kg) + Melatonin (20 mg/kg; simultaneous), G- Kainic acid (45 mg/kg) + Melatonin (5 mg/kg; pre-treatment), H- Kainic acid (45 mg/kg) + Melatonin (20 mg/kg; pre-treatment).

**Figure 4:** Liver tissue mtDNA Cytochrome b sequence *in vivo*. Ref seq- Reference sequence. A- Normal control (without treatment), B- Melatonin (20 mg/kg), C- Kainic acid control (45 mg/kg), D- Kainic acid (45 mg/kg)+Melatonin (5 mg/kg; simultaneous), E- Kainic acid (45 mg/kg)+Melatonin (10 mg/kg; simultaneous), F- Kainic acid (45 mg/kg)+Melatonin (20 mg/kg; simultaneous), G- Kainic acid (45 mg/kg)+Melatonin (5 mg/kg; pre-treatment), H- Kainic acid (45 mg/kg)+Melatonin (20 mg/kg; pre-treatment). Analysis is repeated three times.





**Figure 5:** A- *In vitro* effect of melatonin on 3H-Thymidine incorporation by kainic acid treated splenocytes and CD4+lymphocytes. a- Normal control, b- Kainic acid control (1 mM), c- elatonin (1 mM), d- Kainic acid (1 mM)+Melatonin (0.25 mM), e- Kainic acid (1 M)+Melatonin (0.5 mM), f- Kainic acid (1 mM)+Melatonin (1 mM);Total splenocytes (cpm/2×106 cell), CD4+lymphocytes (cpm/8×105 cells). Values are mean ± S.D \*P<0.005 (compared to respective normal control); \$P<0.005, #P<0.05 (compared to Kainic acid control). n=4 in each group. B- *In vivo* effect of melatonin on 3H-Thymidine incorporation by kainic acid challenged splenocyte and CD4+lymphocytes. a- Normal control, b- Kainic acid control (45 mg/kg), c- Melatonin (20 mg/kg), d- Kainic acid (45 mg/kg)+Melatonin (5 mg/kg; simultaneous), e- Kainic acid (45 mg/kg)+Melatonin (10 mg/kg; simultaneous), f- Kainic acid (45 mg/kg)+Melatonin (20 mg/kg; simultaneous), g- Kainic acid (45 mg/kg)+Melatonin (5 mg/kg; pre-treatment), h- Kainic acid (45 mg/kg)+Melatonin (20 mg/kg; pre-treatment). Total splenocytes (cpm/2×106 cell), CD4+ lymphocytes (cpm/8×105 cells). Values are mean ± S.D \*P<0.005(compared to respective normal control); \$P<0.005, #P<0.05(compared to Kainic acid control) \*P<0.005(compared to respective normal control); \$P < 0.005, #P<0.05(compared to Kainic acid control). n=4 in each group

induced liver inflammation by lymphocyte infiltration and increased inflammatory cytokine mRNA expression. Such a state of liver damage may be the manifestation of the oxidative stress. It is likely that KA may damage kidney and other organs too. This is being investigated in the laboratory.

Interestingly, the results of the present study showed that KA markedly decreased the expression of mRNA for IL-10 and drastically increased that for MCP-1 in liver tissue. It also increased the expression of IL-6 and INF  $\gamma$  mRNA. Increased expression of MCP-1 would mediate monocyte infiltration into the liver and the influence of other cytokines could result in intense activation of Kupffer cells, macrophages/monocytes, etc. This could result in the generation of excess free radicals. Thus the present study supports to the hypothesis that immune mediated oxidative stress occurs not only in brain but also in other tissues (liver) and the oxidative stress could be the major mechanism of KA-induced toxicity.

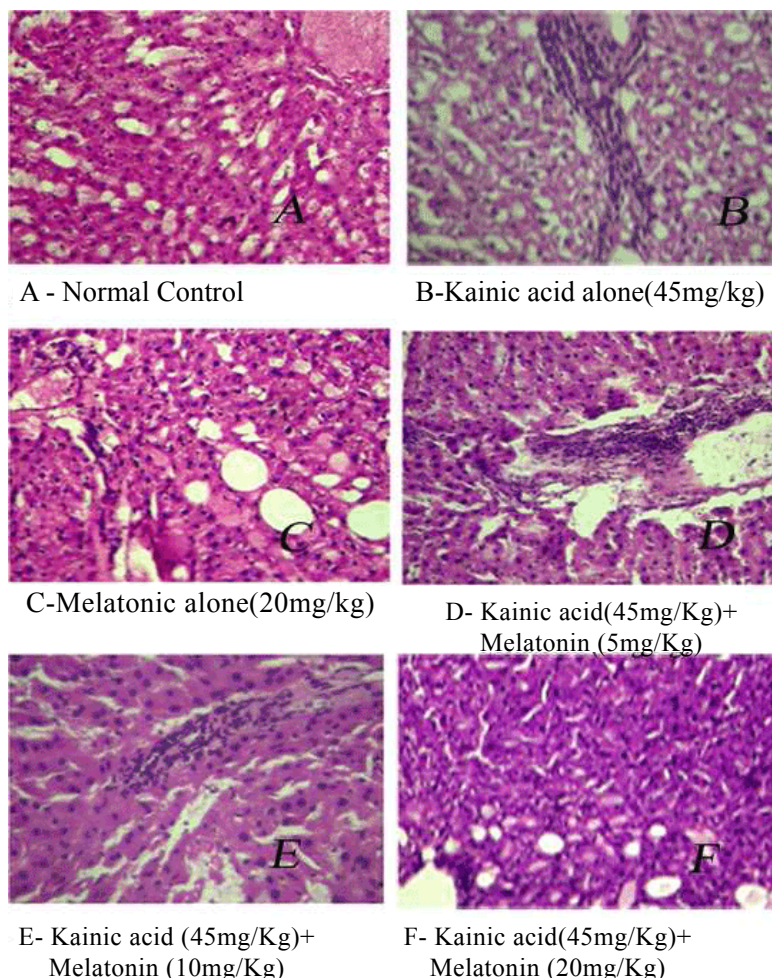
MLT has been reported to have important antioxidant properties. It scavenges reactive oxygen species, lowers hydrogen peroxide levels and restores glutathione homeostasis [42] and activates NK cells and Th2 cells [43]. Moreover, it stimulates the activities of glutathione

reductase and glutathione peroxidase involved in the GSH cycling [44,45]. Further, MLT is known to attenuate KA-induced oxidative stress, neuro-degeneration and DNA damage [46]. In this context, the present study reveals in a pronounced manner that MLT could counteract KA-induced alterations in the expressions of mRNAs for IL-10, IL-6, MCP-1 and INF  $\gamma$  in liver. Thus, it appears that MLT could suppress the toxicity of KA, at least partly, by preventing or reducing KA-induced alterations in the expression of these cytokines / chemokines in liver.

MLT has several actions on the immune system [47]. It could engineer the growth of CD4+ cells [48]. It promotes Th2mediated immune response including production of IL-10 [49]. Furthermore, MLT down regulates Th1 cytokines such as IL-6 and INF- $\gamma$  [50]. In the present study, KA down regulated IL-10 and up regulated IL-6 and INF- $\gamma$ . These changes were completely counteracted by MLT.

It is well reported in literature that IL-10 follows the pathway of Th2 cells whereby activating MHC-I and inhibiting Th1 cells. Since MLT counteracted KA-induced reduction in IL-10 expression, it is likely that it inhibits Th1 mediated stimulation of reactive oxygen species production also.





**Figure 6:** Histopathological evaluation on the effect of melatonin on kainic acid- induced liver toxicity (Hematoxylin and Eosin stained sections, 400X) – a- Control - of liver showing normal architecture and mild fatty change only, b- Kainic acid alone (45 mg/kg)- marked perivascular lymphocytic infiltrate, granular cell change and ballooning degeneration of hepatocytes, c- Melatonin Alone (20 mg/kg)- normal architecture and mild fatty change only, d- Kainic acid (45 mg/kg)+Melatonin (5 mg/kg) - normal architecture, mild fatty change and mild perivascular lymphocytic infiltrate, e- Kainic acid (45 mg/kg)+Melatonin (15 mg/kg)- normal architecture and mild perivascular lymphocytic infiltrate, f- Kainic acid (45 mg/kg)+Melatonin (20 mg/kg)- showing normal architecture and mild fatty change only.

Melatonin counteracted the changes in GSH induced by KA in cultured cerebellar granule neurons. Melatonin prevented the neurotoxic effects of reactive oxygen species linked to KA receptor activation by maintaining cellular GSH homeostasis [51]. Our results also show that MLT counteracted the elevation of pro inflammatory markers induced by KA.

There is extensive evidence that oxidative stress mediated damage occurs to lipids of cellular membranes, proteins and DNA. In nuclear and mitochondrial DNA, 8-hydroxy-2'-deoxyguanosine (8-OHdG) or 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxodG) is one of the predominant forms of free radical-induced oxidative lesions, and has, therefore, been widely used as a biomarker for oxidative stress and carcinogenesis [52]. Liver cytochrome b mitochondrial genome DNA sequence analysis revealed repeated base mismatch in KA alone treated group (*in vivo*) which may be due to KA-induced oxidative damage to DNA as well as the increase in 8OHdG formation *in vitro* and *in vivo* studies supported this finding. KA induced oxidative damage of DNA which was inhibited by co treatment of MLT in a concentration dependent manner.

MLT reduce the extent of cell damage associated with pathologies such as epilepsy that involve the activation of kainate-sensitive glutamate receptors [53,54]. Our results suggest that MLT might reduce the extent of cell damage (DNA adduct and mtDNA) associated with pathologies in case of liver damage that involve the activation of kainate-sensitive glutamate receptors which may be present in liver. Melatonin's ability to reduce KA-induced increases in hepatic 8-OH-dG levels presumably relates to its direct free radical scavenging ability and possibly to other antioxidative actions of melatonin.

## Conclusion

The study clearly depicts that KA induces oxidative damage to liver mtDNA in the form of DNA adducts and base mismatch which may lead to mutations and altered protein synthesis in due course of time. It is of interest to note that MLT has a protective action on the damage caused by KA by protecting the DNA from base alterations. Hence MLT can be used as a therapeutic agent against liver toxicity.

## Competing Interests

There is no competing interest among authors.

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